Transactions of the Royal Society of Tropical Medicine and Hygiene (2008) 102/S1, S5–S9

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Soil physicochemical properties related to the presence of *Burkholderia pseudomallei*

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KEYWORDS
Melioidosis
Burkholderia pseudomallei
Soil
Environment
Physicochemical properties
Thailand

Summary The incidence of *Burkholderia pseudomallei* in the soil from north-east Thailand is estimated to be 20-fold higher than that from central Thailand, and is associated with a 10-fold higher incidence of melioidosis in the region than in central Thailand. This study investigated the presence of *B. pseudomallei* in relation to the physicochemical properties of soil from Khon Kaen province, north-east Thailand. Thirteen districts (54.2%) were positive for *B. pseudomallei*. From a selected district, *B. pseudomallei* was cultured from 19 of 50 sites (38%). The soil in this area was predominantly sandy. From the positive sites, the organism was found mainly at a depth of 30 cm (43/68, 63% of isolates) and was significantly associated with certain soil physicochemical parameters, including a pH of 5.0–6.0, a moisture content >10%, and higher chemical oxygen demand and total nitrogen than negative sites ($P < 0.05$). *Burkholderia pseudomallei* is unevenly distributed in this area, with the pH of the soil being the major determinant of the presence of the organism. The sandy soil type of north-east Thailand can support the survival of *B. pseudomallei* and allow it to move freely with water flow, and thus readily come in contact with people during the rainy season.

1. Introduction

Burkholderia pseudomallei, a Gram-negative bacillus, is the causative agent of melioidosis, a fatal infectious disease endemic to Southeast Asia and northern Australia. Numerous sporadic cases are also reported on every continent.[1–3] The infection occurs through inhalation, or skin abrasions coming into contact with contaminated soil or water. In a clinical trial, patients with *B. pseudomallei* sepsis were associated with a mortality rate of approximately 40%.[4] However, this figure is an underestimate as the study excluded septic-shock patients who died before being enrolled in the programme.

Smith et al. (1995)[5] estimated the amount of *B. pseudomallei* in the soil from north-east Thailand to be 20-fold higher than that from central Thailand. This high incidence of *B. pseudomallei* is associated with a 10-fold higher incidence of melioidosis in the region[6] than in central Thailand. In addition, the ratio of *B. pseudomallei* to *B. thailandensis* (a closely related, non-pathogenic bacterium) is higher in the north-east[5,7] than in central Thailand. Some environmental factors, such as pH and salt (osmotic stress), have been shown to affect the survival of the organism in the laboratory.[8] The direct measurement of the physicochemical properties of soil may thus be able to predict the presence of *B. pseudomallei*. The goal of this study was to relate soil physicochemical properties to the presence or absence of *B. pseudomallei* in the endemic area of north-east Thailand.

2. Materials and methods

2.1. Soil survey

From September to December 2002, five soil samples per district were collected from 24 districts all over Khon Kaen province (10,886 km^2) in north-east Thailand to investigate

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the presence of *B. pseudomallei*. One district, Nam-Phong, in which 50% of sites were positive for *B. pseudomallei*, was selected for the soil physicochemical property study. Soil samples for this part of the study were collected from all villages of the district from April to September 2004. This time period included summer and the rainy season. During the rainy season soil samples were collected at least 2 days after any rainfall to allow excess water to drain away.

2.2. Soil sampling

Soil sampling at each site in the province was done at a depth of 30 cm by taking a sample from each of three holes arranged in a triangle 1 m apart using a 5″ diameter hand auger. The three samples were pooled to give one representative sample of the site. Soil samples for the selected district of Nam Phong were collected in the same way along both sides of the inter- and intra-village roads at depths of 15, 30 and 45 cm. Samples from the same depth were pooled to obtain at least 500 g of soil. Soil from each depth was used for bacterial culture, while for identifying soil physicochemical properties, only samples from a depth of 30 cm were used. Each soil sampling site was located by a global positioning system (GPS) device (Trimble Navigation, Sunnyvale, CA, USA). The site coordinates were plotted in the topographic map (Figure 1) using ArcGIS 9.1 software (ESRI, Redlands, CA, USA).

2.3. Bacterial isolation and identification

Soil from each sample (3 g) was vigorously mixed with 3 ml distilled water and left for 30 min to allow sedimentation. Five hundred microlitres of the supernatant were transferred into 3 ml of selective enrichment broth (threonine-basal salt solution with 20 μg/l colistin).[5] After incubating at 42°C for 48 h with shaking, 100 μl of broth in dilutions of 10^{-2}, 10^{-3} and 10^{-4} were spread on modified Ashdown's agar[9] and incubated at 42°C for 4 days. The culture plates were examined every day and suspected colonies (purple–pink, smooth or wrinkled) were counted and picked up for identification by biochemical tests, including the assimilation of L-arabinose (*B. pseudomallei* is negative). All *Burkholderia* spp. isolates were confirmed by species-specific real-time PCR.[10] Sites were declared positive if the organism could be isolated from at least one depth at that site.

2.4. Physicochemical properties of soil

pH
Soil (1 g) was mixed vigorously in 2 ml sterile distilled water and the pH of the supernatant measured by pH meter (Thermo Fisher Scientific Inc., Franklin, MA, USA).

Moisture content
Soil samples were collected and stored in zip-locked bags and 2 g of each sample were weighed immediately after transportation from the field using an accurately weighed dish. The soil samples were then dried in an oven at 130°C for 2 h and cooled in a desiccator. Finally, the dried soil was weighed. The moisture content was calculated as the weight difference between the original and dried samples, expressed as a percentage of the original weight of the sample.

Chemical oxygen demand
The chemical oxygen demand (COD), providing an indirect measure of the humus (organic carbon) content of the samples, was analyzed by the dichromate oxidation method.[11]

Total organic nitrogen
The total organic nitrogen content of the soil samples, indicating fertiliser use, was measured by the Kjeldahl distillation method (total Kjeldahl nitrogen [TKN]) using 0.7–2.2 g of soil.[12]

Grain-size distribution
The grain-size distribution of the samples was determined by using sieve analysis, which classifies the soil by whether it is predominantly gravel, sand or fine particles (silts and clay).[13] The oven-dried soil was broken up with a rubber hammer to separate the particles. 200 g of soil were processed through a sieve shaker unit (Acquavir International, Mumbai, India) for 10–15 min. The soil residues left in each sieve unit were weighed and classified according to the grain-size distribution curve.

Nitrate analysis
The amount of nitrate in 1 g soil in 5 ml de-ionized water was analyzed by a cation–anion measurement apparatus (Orion-940 pH/ISE Meter, Thermo Electron Corporation, Waltham, MA, USA) using a nitrate probe.

2.5. Statistical analysis

The soil physicochemical factors that correlated with the present of *B. pseudomallei* were calculated by the independent *t* test.

3. Results

A total of 344 soil samples were collected from 24 districts of Khon Kaen province. Thirteen districts (54.2%) were culture positive for *B. pseudomallei* and five (20.8%) for both *B. pseudomallei* and *B. thailandensis*. In only four districts were soil samples from at least 50% of sites positive for *B. pseudomallei*. Among positive soil sites 64.1% of samples were positive for *B. pseudomallei* and 35.9% were positive for *B. thailandensis*.

Nam Phong, a district with 50% of sites positive for *B. pseudomallei*, was selected for further soil surveys. The terrain of this district is covered mainly by the flood plain of the Nam Phong River. The major land uses are rice paddy fields in lowland areas and some cassava fields in the uplands. It is one of the most fertile districts of the region and the majority of the population work in close contact with the soil during the planting season.

Fifty soil samples were collected from 50 villages (sites) covering an area of 50 km^2. *Burkholderia pseudomallei* was cultured from 38% of the collection sites (19/50). The positive sites were mainly rice fields located near water sources (Figure 1). From the 19 positive sites, soil samples from depths of 15 and 30 cm were 96% and 100% positive, respectively, for *B. pseudomallei*, while from 45 cm only 5% were positive. Among all depths at positive sites a total of 68 isolates of *Burkholderia* spp. were obtained. Six isolates of *B. thailandensis* were identified, of which five were found at the same depth at the same site as

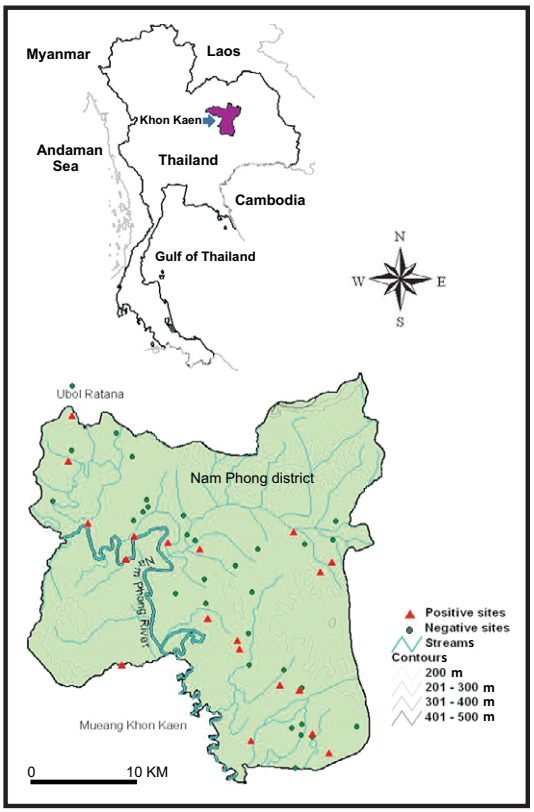

Fig. 1 – Map of Nam Phong district in Khon Kaen province, north-east Thailand with positive (triangles) and negative (circles) soil sites labelled. Top panel: a map of Thailand, neighbouring countries and the location of Khon Kaen province.

Table 1 The mean values of soil pH, moisture content, total nitrogen, chemical oxygen demand (COD) and nitrate concentration at negative (31) and positive (19) *Burkholderia pseudomallei* sites

Soil site	pH	Moisture content (%)	Total nitrogen (mg/kg)	COD (mg/kg)	Nitrate (ppm)
Negative	6.4[a]	7.77[a]	22.75[a]	2349[a]	38.1
Positive	5.6[a]	14.92[a]	37.13[a]	3435[a]	38.5

[a] Statistically significant difference ($P < 0.05$) between positive and negative soil sites.

B. pseudomallei and another was found at a different depth at the same site. Upon sub-culture from a single colony on Ashdown's medium, phenotypic variation, i.e. size (small, medium and large) and colour (dark purple or paler), was observed. Only 10% of the sub-cultured isolates with different phenotypes showed different ribotype patterns (data not shown).

The grain-size analysis showed that the majority (76%) of the soil samples from the study area were sandy. The humus (organic carbon) content of soil was measured indirectly by COD, and the use of nitrogen fertilizer was indicated by nitrate and total nitrogen. Soil at a depth of 15 cm had the highest COD (3475 mg/kg), total nitrogen (53.52 mg/kg) and nitrate compared with other depths, as expected, as it is the level of soil where fertilizer would have been incorporated. The soil physicochemical properties that were significantly correlated with the presence or absence of *B. pseudomallei* were a pH of 5.0–6.0, a moisture content >10%, and a higher COD and total nitrogen in positive than negative sites ($P < 0.05$) (Table 1 and Figure 2).

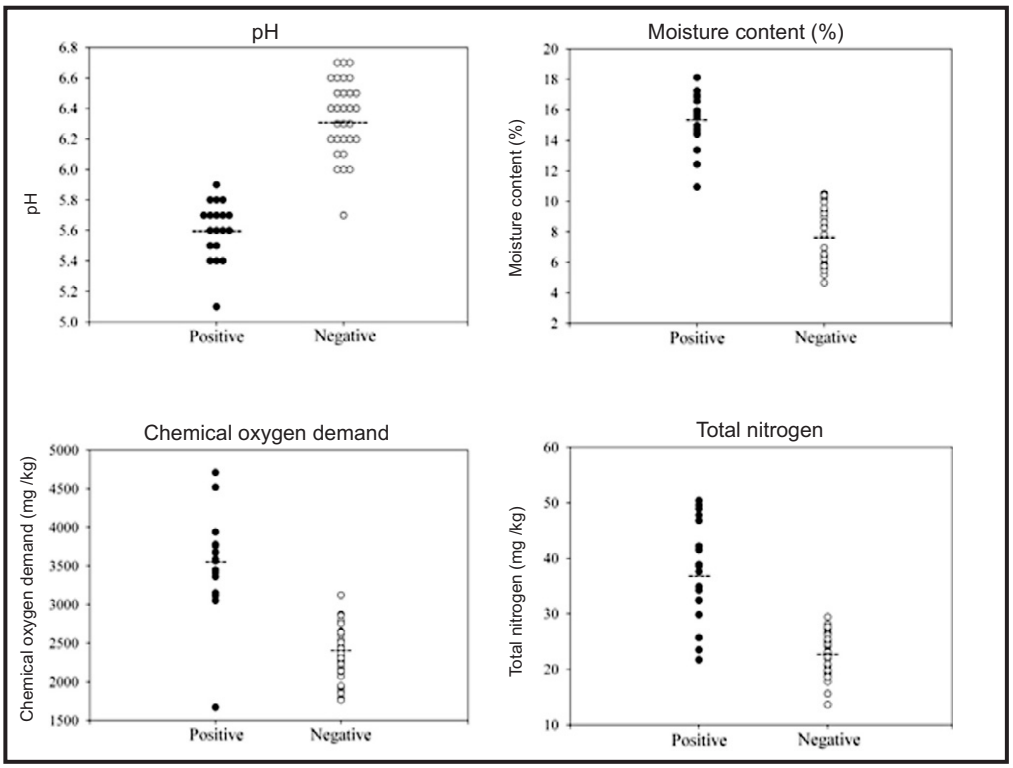

Fig. 2 – Scatter plot of pH, moisture content, chemical oxygen demand (COD) and total nitrogen values at negative and positive soil sites. The horizontal lines indicate the mean value of each parameter.

Although soil collection was performed both in summer (64% of sites) and in the rainy season, the mean moisture content examined at the same depth (30 and 45 cm but not 15 cm) from both seasons was not significantly different (data not shown). Moreover, the organism was found to be predominantly located at a depth of 30 cm in both summer and the rainy season.

4. Discussion

The environmental factors that support the survival of *B. pseudomallei* in soil and affect the way humans become infected more frequently during the rainy season remain to be elucidated. Soil in the north-east of Thailand is mainly sandy, as were 76% of the soil samples investigated in this study, while soil in central Thailand contains more humus and more clay.[14] The proportions of *B. pseudomallei* and *B. thailandensis* present in soil in Khon Kaen province were similar to those reported for north-east Thailand by Wuthiekanun et al. (1995).[15] However, in the selected area investigated in this paper, only 8.8% (6/68) of *Burkholderia* isolates were *B. thailandensis* and were located at the same depth or the same site as *B. pseudomallei*. This may indicate that the same soil factors equally promote the survival of both species but cannot explain the unequal distribution of *B. thailandensis* and *B. pseudomallei* in each region of Thailand.[7]

The phenotypic variation we observed during single-colony sub-culture of *B. pseudomallei* has also been observed in other studies.[16,17] We observed variation between colonies in terms of size and colour rather than in overall morphological characteristics, even with repeated sub-culture. Chantratita et al. (2007)[18] reported seven types of colony morphology from sub-culture of a clinical isolate. However, the most common morphology of our soil isolates was not similar to any of them. A shift from environmental conditions to laboratory conditions may not alter the gene expression profile, and thereby affect the morphology, as much as removing the organism from the human body to laboratory conditions. It may be of interest to observe the morphology of soil isolates after passing through an animal model. To confirm that differences in size or colour of colonies were not due to contamination, their ribotype patterns were observed and found to be the same as their paired samples. However, the genotypic variation among these soil isolates was very low, as indicated by ribotyping and later confirmed by multiple-locus variable-number tandem repeat analysis and multilocus sequence typing[19] Only four ribotype patterns were found, compared with 77 types reported for *B. pseudomallei* isolated from all over Thailand.[20] The environmental niche in this area may select for the presence and expansion of these ribotypes, as suggested by other populations of *B. pseudomallei*.[21]

The majority of the organisms were found at a depth of 30 cm both in summer and the rainy season. The loosely packed sandy soil may allow the organism to move rapidly up and down with the water level so it does not persist close to the soil surface. Therefore, the association of melioidosis cases with the rainy season may be related to ploughing of the contaminated land beyond a depth of 30 cm during the planting season. Moreover, the sandy soil may explain why north-east Thailand has no reports of outbreaks after heavy rainfall, unlike in Hong Kong,[22] or after summer rain as occurs in northern Australia.[23]

A soil water content of 20% was reported to support *B. pseudomallei* survival for more than 1 year under laboratory conditions, while a moisture content of 10% only allowed 70 days' survival.[24] The moisture content of the soil at all the positive sites in this area was in the range 9–18%. Humus in soil (organic matter) may help support the survival of the organism where there is low moisture content. There was one positive site with a moisture content of 9% (the rest were more than 10%) but the COD and total organic nitrogen were relatively high. Conversely, there was one positive site with low COD and total nitrogen (1500 mg/kg and 25 mg/kg, respectively) but with high moisture content at 18%. Continuous investigation of these parameters in soil, and isolation of bacteria, will help to confirm the survival period of the organism in this range of moisture. At a depth of 45 cm the moisture content was low and the COD value implied that the humus content was also quite low. This may explain why only few bacteria were isolated at this depth or suggests that they were in an unculturable form.[8] These data are not consistent with an intensive soil sampling in northern Australia, where the samples were from clay soil. Samples collected at depths of 25–45 cm were reported to be the most likely to yield isolates of the organism.[25] It has been proposed that sandy soil is less able to support the long-term persistence of *B. pseudomallei* than heavy clay soil[26]; however, our study demonstrated that sandy soil can also support the survival of the organism, although the important factors here may be the moisture content and nutrient availability of the soil in each area rather than the depth and type of soil. It was demonstrated under laboratory conditions that *B. pseudomallei* can survive for years at pH 5–8. In soil, the pH range from 5.1 to 5.9 is associated with the presence of *B. pseudomallei*, and all except one of the negative sites had a pH >6.0. This finding confirmed the report of Kanai and Kondo (1994)[27] that the endemic area for melioidosis in north-east Thailand has unusually acid soil conditions. This may be the most crucial physicochemical factor associated with the presence of *B. pseudomallei* and perhaps also for the presence of *B. thailandensis*.

The information obtained from this study indicates that *B. pseudomallei* in the soil in Nam Phong district is unevenly distributed across the area. The sandy soil allows the organism to move more easily with water flow than heavy clay soil, which tends to trap water and promote biofilm formation. The abundance of the organism in the north-east allows it to come in contact with susceptible hosts during the rainy season and cause melioidosis. However, soil type alone cannot explain the higher number of organisms found in soil of the north-east, or the lower proportion of *B. thailandensis*. The soil physicochemical parameters and the presence of other organisms in the soil may participate as well. Thus, the finding that some soil physicochemical properties correlated with the presence of *B. pseudomallei* may be useful to control or limit the incidence of this fatal disease.

Authors' contributions: RWS and RL designed the study protocol; RWS and SW analyzed and interpreted the data and designed the protocol for bacterial culture and

identification; SP carried out the soil sampling located by GPS positioning, the physicochemical examination and bacterial culture from soil; PR carried out the statistical analysis and prepared the topographic map; RWS drafted the manuscript. All authors read and approved the final manuscript. RWS is guarantor of the paper.

Acknowledgements: We are very grateful to Emeritus Prof. James A. Will, Department of Pathobiology, SVM and Surgery, School of Medicine and Public Health, University of Wisconsin-Madison, USA for editing the English of the manuscript.

Funding: This project was supported by a grant from the Commission on Higher Education, Thailand.

Conflicts of interest: None declared.

Ethics approval: Not required, as recommended by Khon Kaen University ethical committee.

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